EFFECT OF IRRADIATION OF ULTRAVIOLET ON THE QUANTITATION METHOD OF ENHANCED GREEN FLUORESCENT PROTEIN

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ABSTRACT

In last 30 years, green fluorescent protein (GFP) has changed from an unknown protein to a commonly used protein in bioscience application due to its visible fluorescence. As the usage of GFP increases, fluorescent detection and measurement devices are becoming more important. To detect and measure the GFP, a gel-based imaging system using a native polyacrylamide gel was developed. The ultimate aim of this study is to investigate the effect of ultraviolet (UV) light on the GFP quantitation method. In the research, enhanced GFP (EGFP) was expressed in *Escherichia coli* strain BL21(DE3) and purified using immobilized metal ions affinity chromatography. Different dilution of EGFP was prepared and their concentrations were determined by Lowry's method using bovine serum albumin as the standard. The EGFP dilution samples were then loaded into a native polyacrylamide gel. After electrophoresis, fluorescent image of EGFP on the gel was captured using gel imaging system under different UV irradiation exposure period. The UV irradiation has a marked influence on the EGFP fluorescence intensity. The fluorescence intensity was increased as the UV exposure period increased from 5-35 s. However, the fluorescence intensity decreased when the exposure period was increased further. Highest fluorescence intensity happened at around 35 s of UV exposure. By using different concentration of purified EGFP, the photobleaching process followed a first order reaction with rates between 3712-8213 int/s. The linearity showed insignificant change and lied within 0.922-0.946. It became more reliable when the UV exposure time increases. However, UV exposure time affected the fluorescence intensity, it is better to choose around 35s as UV exposure time due to highest fluorescence intensity when using gel-based imaging method as quantitation method.

Key words: GFP, EGFP, UV, gel-based imaging method, quantitation

ABSTRAK

Dalam 30 tahun yang lalu, protein perndarfluor hijau (GFP) telah berubah daripada protein yang tidak diketahui kepada protein yang biasa digunakan dalam kegunaan biosainas kerana sifatnya yang boleh bercahaya hijau dan dapat dilihat. Oleh sebab kenaikan penggunaan GFP, alat pengesanan dan pengukuran telah menjadi semakin penting. Untuk mengesan dan mengukur GFP, system pengimejan yang berasaskan gel dengan menggunakan gel polyacrylamide asli telah dibangunkan. Matlamat utama kajian ini adalah untuk mengkaji kesan cahaya ultraugu (UV) pada kaedah kuantiti GFP. Dalam kajian ini, enhanced GFP (EGFP) telah ditunjukkan dalam Escherichia coli strain BL 21 (DE 3) dan disucikan dengan mengunakan ion logam bergerak pertalian kromatografi. Pencairan EGFP yang berbeza telah disesdiakan dan kepekatan mereka telah ditentukan dengan oleh kaedah Lowry dengan menggunakan albumin serum lembu sebagai standard. Sample pencairan EGFP kemudian diisikan ke dalam gel polyacrylamide asli. Selepas elektroforeis, imej pendarflour EGFP yang berada dalam gel ditangkap gambar di bawah sinaran UV dalam tempoh pendedahan yang berbeza. Sinaran UV telah menpengaruhi intensiti pendarfluor EGFP. Intensiti pendarfluor EGFP telah ditingkatkan apabila tempoh pendedahan UV telah dinaikan dari 5 hingga 35 s. Walaubagaimanapun, intensiti perdarflour menurun apabila tempoh pendedahan itu telah meningkat. Intensiti pendarfluor yang paling tinggi ialah 35 saat apabila didedahkan oleh sinarran UV. Dengan menggunakan pelbagai kepekatan EGFP yang telah disucikan, proses ‘photobleaching’ diikuti tindak balas tertib pertama dengan kadar antara 3712 – 8213 int/s. Kelinearan menunjukkan perubahan yang tidak ketara dan berada dalam 0.922-0.946. Kelinearan itu menjadi lebih linear apabila masa pendedahan UV meningkat. Walaubagaiamanapun, masa pendedahan UV menjejaskan intensity pendarfluor. Oleh itu, masa 35s adalah lebih baik dipilihkan sebagai masa

pendedahan UV kerana intensity pendarfluor adalah paling tinggi apabila menggunakan kaedah berasaskan gel sebagai kaedah quantitation.

Kata kunci: GFP, EGFP, UV, kaedah pengimejan berasaskan gel, kuantiti

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LIST OF ABBREVIATIONS

APS	-	*Ammonium persulfate*
A.victoria	-	*AequoreaVictoria*
BSA	-	*Bovine serum albumin*
C.elegans	-	*Caenorhabditiselegans*
DNA	-	*Dioxyribonucleic acid*
E.coli	-	*Escherichia coli*
EGFP	-	*Enhanced green fluorescent protein*
GFP	-	*Green fluorescent protein*
IPTG	-	*Isopropyl- β-D-thiogalactoside*
LB	-	*Luria bertani*
NA	-	*Nucleic acid*
n-PAGE	-	*Native-polyacrylamide gel electrophoresis*
OD	-	*Optical density*
PAGE	-	*Polyacrylamide gel electrophoresis*
RNA	-	*Ribonucleic acid*
R.reniformis	-	*Renillareniformis*
Temed	-	*Tetramethylethylenediamine*
UV	-	*Ultraviolet*
wtGFP	-	*Wild type green fluorescent protein*
D-D	-	*Dye to dye*
D-O	-	*Dye to oxygen*
QD	-	*Quantum Dots*

1 INTRODUCTION

1.1 Background of green fluorescent protein

Fluorescent proteins can be found from the mostly marine creatures. Green fluorescent protein (GFP) is originated from the bioluminescent jellyfish which also known as *Aequorea victoria (A. victoria)* in the sea of north pacific (Zimmer, 2002; Chalfie *et al.*, 1994). GFP is a 27 kDa protein which is made of 238 amino acids polypeptide and composed of 11- strand β sheet with a central coaxial α helix in a novel 3-D configuration (Yang *et al.*, 1996; Ormo *et al.*, 1996). GFP chromophore is lied within the β sheet. This chromophore is formed when tri-peptide,-(Ser_{56}-Tyr_{66}-Gly_{67})-,of GFP is going through cyclisation, oxidation and dehydration reactions (Yang *et al.*, 1996; Ormo *et al.*, 1996). The chromophore is the source of green light where it absorbs light energy from the ultraviolet (UV) and emits a low energy green light. This phenomenon happens when the Ca^{2+} ions react with Aequorin. Nowadays, there are many types of GFP derivatives and the basic form of the GFP is the wild type GFP (wtGFP). The wtGFP with 238 polypeptides is stable and proteolysis-resistant. It has the excitation peak at 395 nm and a minor peak at 475 nm (Ward *et al.*,1980). GFP has been modified into different type of GFP derivatives like enhanced GFP (EGFP) and S65T by modifying certain location of amino acid. Random mutagenesis affects the proteins spectral characteristic, hence these mutant GFPs has a more powerful green fluorescence intensity when are excited at specific absorbance (Philip, 1997). In this study, EGFP will be used as the model protein due to its better fluorescence properties compared to GFP.

1.2 Motivation and problem statement

Around 30 years ago, the GFP was discovered by Osamu Shimomura and this discovery was further developed into many applications which are important and useful in the science life today. These applications including protein markers, tag for protein localization, and protein-protein interactions. In 2008, the Royal Swedish Academy of Sciences had awarded the Nobel Prize to Osamu Shimomura, Martin Chalfie and Roger Yonchien Tsien for the discovery and development of the GFP (Tongea and Meechb, 2009; Nienhaus, 2008). Given its large number of applications, the reliable quantitation methods such as spectrofluorometer, flow cytometry, fluorescent microscopy and gel-based imaging system are designed to analyze the GFP samples. In this research, gel-based imaging system is used for GFP quantitation. This analytical method only required microgram amount and small volume of samples for the analysis. Furthermore, gel-based imaging system is able to quantify the denatured GFP from its native form (Chew *et al.*, 2011). Gel-based imaging system uses UV lamps as the illumination source for green fluorescent detection. The UV radiation may affect the reproducibility and accuracy during the quantitation. Prolonged irradiation of UV on GFP may induce photoconversion in the chromophore (Patterson *et al.*, 1997). It causes initial increase in the fluorescence and photobleaching effects on different type of mutant GFPs. Patterson (2007) has reported that the EGFP photobleaching rate was increased rapidly when it was exposed under the high power of UV light for a long period. Thus, the exposure time effect of the UV irradiation on GFP fluorescence is crucial for reliable and accurate GFP quantitation using the gel-based imaging method.

1.3 Objectives

This research study was to investigate the effect of UV irradiation period on different concentration of purified EGFP quantitation using gel-based imaging method.

1.4 Scope of this research

The following are the scopes of this research:

(i) Expression of EGFP in *E.coli* strain BL21 (DE3)

(ii) Purification using affinity chromotagraphy.

(iii) Determination of the amount of EGFP by using Lowry's method.

(iv) Investigation of the effect of UV irradiation period and EGFP concentration on the quantitation method.

2 LITERATURE REVIEW

2.1 Properties of green fluorescent protein

GFP is from marine creatures: a jellyfish, *Aequorea victoria*, from North-west pacific and a sea pansy, *Renilla reniformis*, from Georgia coastline (Ward *et al.*, 1980). Although both *Aequorea* GFP and *Renilla* GFP share the identical chromophore, *Aequorea* GFP has two absorbance peaks at 395 and 475 nm while *Renilla* GFP has only a single absorbance peak at 198 nm (Deluca and Mcelroy, 1981). Besides, *Renilla* GFP has 5.5-folds greater monomer extinction coefficient than the *Aequorea* GFP which has a 395 nm peak absorbance (Deluca and Mcelroy, 1981). Hence, only *Aequorea* GFP genes are cloned for various application (Tsien, 1998). GFP is an acidic, compact, globular protein with 27 kDa molecular weights (Chalfie and Kain, 2005). Table 2-1 and Table 2-2 show the comparisons of the physical properties and the amino acid compositions of the *Aequorea* GFP and *Renilla* GFP.

Table 2-1**:** Comparisons of the physical properties of the Aequorea GFP and Renilla GFP [Adapted from Chalfie and Kain (2005)]

	Aequorea	*Renilla*
Monomer molecular weight[a]	27 kDa 26.9 kDa[c]	27 kDa[b]
Isoelectric point(s) (pI)	4.6–5.1[d]	5.34 ± 0.07[b]
Fluorescence emission maximum	508[e]–509 nm	509 nm[b]
Fluorescence quantum yield	0.72–0.78[e] 0.80	0.80[b]
Molar extinction coefficient (monomer) $\varepsilon\lambda^{1M}$ (liter mol^{-1} cm^{-1})		
λ = 498 nm	3,000	133,000[b]
λ = 475 nm	14,000	53,000[b]
λ = 397 nm	27,600	<1,000[b]
λ = 280 nm	22,000	22,000[b]
Absorption ratio (highest purity achieved) 498 nm/280 nm		5.6[b]–6.0
397 nm/380 nm	1.25	

[a] At moderate protein concentration of *Aequorea* GFP (<0.5 mg mL^{-1}) the monomeric form predominates. At higher protein concentrations of *Aequorea* GFP (>2.0 mg mL^{-1}) the dimeric form predominates. Renilla GFP is dimeric (2 × 27 kDa) at all concentrations unless denatured. [b] From Ward and Cormier (1979). [c] From Prasher et al. (1992). Based upon sequence of cDNA. [d] From Cutler (1995). Nine isoforms have been characterized. [e] From Morise et al. (1974).

Table 2-2: The amino acid compositions of Renillas GFP and Aequorea GFP [Adapted from Chalfie and Kain (2005)]

Amino Acids	*Renilla* GFP Nearest Integer per 27,000 Da[a]	*Aequorea* GFP from cDNA Sequence gfp 10[b]
Lysine	19	20
Histidine	8	10
Arginine	7	6
Half-cystine	2[c]	2
Methionine	9	6
Aspartic acid	20	18
Asparagine		13
Glutamic acid	27	16
Glutamine		8
Threonine	17	15
Serine	15	10
Proline	11	10
Glycine	22	22
Alanine	14	8
Valine	18	17
Isoleucine	14	12
Tyrosine	11	11
Phenylalanine	13	13
Tryptophan	0[d]	1
Amino sugars	0[e]	0

[a] From Ward and Cormier (1979). Each value represents the average from hydrolyses of 24, 48, and 72 h unless otherwise indicated.
[b] From Prasher et al. (1992).
[c] Determined as cysteic acid following performic acid oxidation.
[d] Determined by hydrolysis in the presence of thioglycolate.
[e] Determined by hydrolysis with *p*-toluenesulfonic acid.

2.2 The formation and mechanism of GFP chromophore

GFP is made of 238-amino acid polypeptides which consists of β barrel with 11 strands GFP that surrounding α helix in a cylindrical structure (Yang *et al.*, 1996; Ormo *et al.*, 1996;McRae *et al.*, 2005). This cylindrical structure is named as 'β-can' which has the function to protect the chromophore that position in the middle of the α helix (Phillips, 1997). Water molecules can form 'stripes' around the cylinder surface and give resistance and stability for chromophore from being unfold caused by denaturants and heat (Phillips, 1997). The α helix contains p-hydroxybenzylideneimadazolinone chromophore which undergoes cyclization of tripeptide (Ser65, Tyr66 and Gly67) and 1,2-dehydrogentaion of the tryrosine (Cody *et al.*, 1993). Based on Figure 2-1, when the translated apoprotein evades

precipitation into inclusion bodies, cyclization of amino group, Gly-67 to the carbonyl group, Ser-65 is occurred to form an imidazolidin-5-one, where the absence of O_2 would stop the process. Then, the new N=C double bond would further to cause dehydrogenation to form a conjugated chromophore (imidazolidin-5-ones). The conjugated chromophore will change to the chromophore completely by undergoing autoxidative formation of double bonds at 4-position. This process needs around one step with a time constant of 4 h (Kidwai and Devasia, 1962).Chromophore is the source of emitting the low energy green light after absorbing the UV light. This phenomenon happens when the Ca^{2+} ions react with Aequorin. The Aequorin which emits the blue light will become an intermediate molecule that further produce a reaction product named blue fluorescent protein (BFP). The excited BFP will further transfer energy to GFP and causes it moves into excited state and emits the green light.

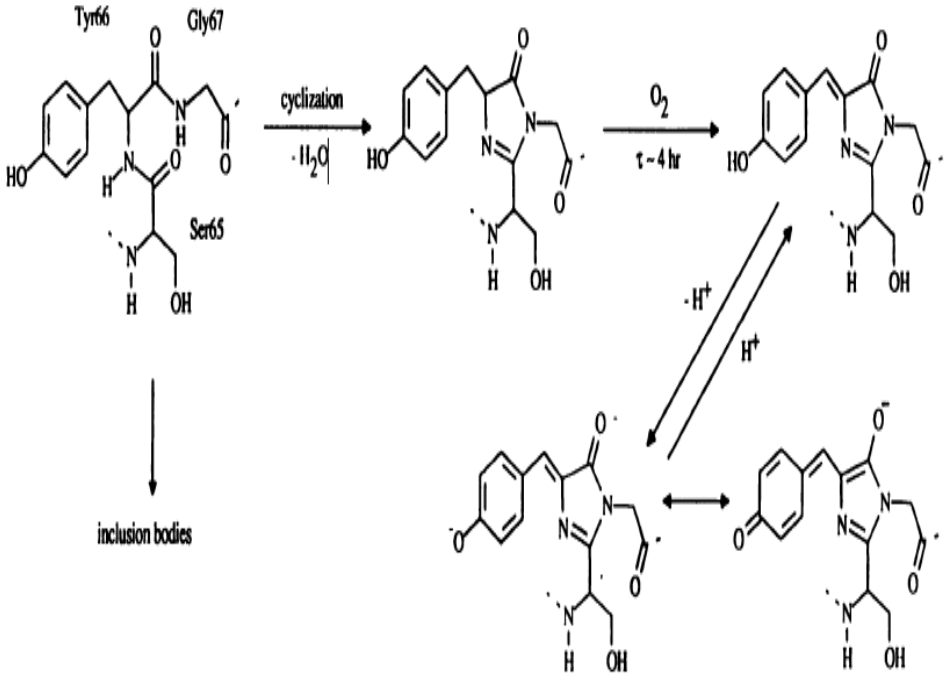

Figure 2-1: The chromophore formation process [Adapted from Heim *et al.* (1994)]

2.3 The derivatives of GFP

GFP is engineering mutated in order to improve its properties. Random mutation is carried out by substituting the certain location of amino acid with other amino acids in the chromophore structure. However, most of the mutations in GFP encountered failures, for example loss of fluorescence without obvious change at certain absorption or emission peaks. The failures are due to the failure formation of chromophore, quenching of the fluorescence and misfolding of the protein (Cubitt *et al.,* 1995). Some successful examples of mutated GFP are S65T and EGFP. For S65T, since the Ser65 is substituted with Thr, it has higher fluorescence intensity, less photobleaching rate, extinction coefficient as well as quantum efficiency compared to wtGFP (Cubitt *et al.,* 1995). For the EGFP, it is a mutant where its fluorescence intensity is increased by 35-fold compared to wtGFP (Cormack *et al.,* 1996). Its enhanced fluorescence intensity causes EGFP becomes so popular in the aspect of the protein marker and reporter (Zhao *et al.*, 1998).

2.4 Applications of green fluorescent protein

2.4.1 GFP as a marker of gene expression and cell lineage

GFP can be used as the gene expression marker in vivo without the needs of the cofactors (Chalfie *et al.,* 1994). This application works when the DNA of GFP is expressed in prokaryotic [*Escherichia coli* (*E.coli*)] or eukaryotic [*Caenorhabditiselegans* (*C.elegans*)] cells (Chalfie *et al.,* 1994). GFP was expressed in the *E.coli* after the induction using Isopropyl- β-D-thiogalactoside (IPTG). Green fluorescence was observed in control bacteria under the illumination UV light. After GFP purification, *the recombinant GFP* exhibits same fluorescence excitation and emission spectra as the purified native protein (Chalfie *et al.,* 1994). This shows that the chromphore of GFP can be formed in the *E.coli* in the absence of other

A.victoria products. As for the *C.elegans,* fluorescent of GFP was produced during the transformation process (Brenner, 1974). Hence, alike with native protein, GFP performs well in term of expression in those cells when illuminated under 450 nm to 490 nm of light.

2.4.2 GFP as a protein tag

GFP can be used as a fluorescent tag for the N- (amino) or C- (carboxyl) termini of proteins (Wang and Hazelrigg, 1994). The ideal fusion of GFP with a host protein preserves both the fluorescence of GFP and all the targeting and physiological function of the host protein. Fusion process happens when both the N- and C-termini are fused with the cytosolic and membrane-bound proteins. The process is functioned successfully without flexible linkers when the amino terminus of GFP is fused at the carboxyl terminus of the host protein. This successful fusion might be enhanced by linker sequences (Cubitt *et al.,* 1995). Application of GFP using as a protein tag is becoming popular. It can be applied in many fields especially in medicine like using GFP to tag lactic acid bacterium strains for live vaccine vectors (Geoffroy *et al.,* 2000).

2.4.3 Monitoring protein-protein interactions

GFP is widely applied in protein-protein interaction application due to its small monomeric reporter molecule that might avoid the obstacles to development of an ideal system to study protein-protein interactions for various applications. (Paulmurugan and Gambhir, 2003).Protein-protein interaction happens when a donor chromophore is fused with an acceptor chromophore such as expressing fusions of two different-colored GFP mutants (Cubitt *et al.,* 1995). For example, blue mutation Y66H acts as the donor, which has maximum excitation of 382 nm and maximum emission of 448 nm while S65T acts as the acceptor which has which

has maximum excitation of 489 nm and maximum emission of 511 nm (Cubitt *et al.,* 1995). This application requires the overlapping between the emission spectrum of the donor and the absorption spectrum of the acceptor (Cubitt *et al.,* 1995). wtGFP is not advisable to be used in this protein-protein interaction because it has the 395nm excitation peak which is almost the same as Y66H which has the 382 nm absorption peak. This will directly excite wtGFP without any energy transfer (Cubitt *et al.,* 1995). Fluorescence resonance energy transfer (FRET) can be used to detect this interaction.

2.5 Quantitation methodss of GFP

There are many analysis methods to detect and measure GFP which including flow cytometry, fluorescent microscopy, spectrofluorometer and gel-based imaging system.

2.5.1 Flow cytometry

Flow cytometry is a technology which is contributive to clinical medicine and cell biology in a very significant manner. Flow cytometry consists of fluidic system, laser, optic system and electronics. Hydrodynamic focusing is applied in flow cytometry (Robinson, 2004). The fluid system transports the samples to the interrogating point that is focused by the laser beam and this produces many optical signals. Flurophores are attached on the cells or particles will emit light when its expose to laser beam at 488nm. Forward scatter (FSC), side scatter (SSC) and fluorescent signals will be collected. FSC is a signal that is used to detect physical size of the particles like cell diameter. SSC is used to detect internal composition, for example red blood cell and white blood cell. Fluorescent signals will follow the same direction as the SSC and pass through a series of short-pass, long-pass and band-pass filters to allow certain wavelength to reach the suitable detectors. Based

on the certain wavelength of the light magnitude, electrical signals are generated and then further analyzed by the computer system. GFP labelled bacteria can be quantified by using flow cytometry. It can quantify fluorescence intensity of various groups of GFP-labelled microorganisms. By using flow cytometry, the quantitation of GFP within a population is allowed because this device is able to analyze the optical properties of hundreds of single cells per second passing through focused laser beam (Errampalli *et al.*, 1999). Flow cytometry is able to identify fluorophores with emission spectra relatively close to each other. Besides, multi-parameter measurements can be obtained concurrently. Additionally, it is able to analyze a huge number of particles in a very short time, however, the generated data will be difficult to be analyzed. Examples of the applications of flow cytometry are transgenic product (GFP), cell viability, cell pigments, DNA and RNA content, chromosome analysis (Castano and Comas, 2012).

2.5.2 Fluorescence microscopy

Fluorescent microscopy is widely applied in the biological sciences due to its magnificent specificity to visualize certain bio-molecule and its ability to study the three-dimensional interior of cells and organisms through fluorescent labeling (Li *et al.*, 2009). The fluorescent microscopy is consists of excitation light source, objective lens,detector, dichroic mirror, emission filter and excitation filter. The fluorescence microscope starts with illuminating the light to let the chromophore absorb the light and cause them to emit low energy light. Then, the microscope has a filter which allows the certain wavelength radiation pass through that matches the fluorescing sample. The radiation will react with the atoms of the specimen and excited the electrons to a higher energy level. They will emit energy when they are in lower level power. The fluorescence that laminated from the sample is separated

from the much brighter excitation light in a second filter, so that it is visible to the human eye. In terms of detecting GFP, the fluorescent microscopic examination is characterized by higher sensitivity. Besides, the spectrofluorometric analysis of cellular lysates reduce screening time to optimize the complementation assay based on reassembly of GFP in order to maximize the percentage of cells showing GFP fragment reassembly (Torrado *et al.*, 2008). The advantage of using fluorescence microscope is that it is able to observe the specific cellular components via molecule-specific labeling and also structures inside a live sample in real time. However, the wavelength of light had limited fundamentally its moderate spatial resolution (Gustafsson *et al.*, 2008). This fluorescence microscope are normally used for the imaging the structural components of organisms like cells, genetic material like DNA and RNA as well as study on the cell populations (Bradbury and Evennett, 1996).

2.5.3 Spectrofluorometer

Spectrofluorometer is used for analysis of fluorescence spectra of liquids, surfaces and glasses (Lakowicz, 2006). The spectrofluorometer consists of a light source (xenon lamp), a sample holder, an excitation monochromator, an emission monochromator and a photo detector (PMT, CCD detector and photodiode) (Lakowicz, 2006).Based on Figure 2-2, a reference sample such as rhodamine is set and it is used to correct for lamp output in order to verify the excitation wavelength as well as correct for difference in detector sensitivity. A high intensity light sources from xenon lamp is then used to cause the maximum molecules inside the sample to become excited state at any one point in time. The light is either passed through an excitation filter or monochromator that enables to select a wavelength of interest to use as the exciting light. The exciting light will pass through the samples and is